EUROPEAN JOURNAL OF DRUG METABOLISM AND PHARMACOKINETICS, 1993, Vol. 18, No. 2, pp. 187–197

Metabolism of S-nicotine in noninduced and Aroclor-induced rats

G. SCHEPERS, K. RUSTEMEIER, R.-A. WALK, and U. HACKENBERG

INBIFO Institut für biologische Forschung, Cologne, Germany

Received for publication: July 17, 1992

Keywords: Nicotine, urinary metabolites, excretion kinetics, Aroclor induction, sex differences, stereoselectivity

SUMMARY

The urinary excretion of nicotine and its metabolites in noninduced and Aroclor-induced male and female rats has been determined following intravenous administration of 2'-[^{14}C]-labeled S-nicotine at a dose of 4.6 μmol/kg. Complete recovery of the administered radioactivity was achieved: 95% in urine and 4% in feces over 96 h and 1% remaining in the body. More than 40 nicotine metabolites were found by radio-HPLC; 19 were identified including the *cis/trans*-diastereomers of nicotine-N'-oxide and 3'-hydroxycotinine. The urinary metabolite profile and excretion kinetics of nicotine and its metabolites were significantly different between noninduced and Aroclor-induced rats. The major urinary nicotine metabolite in the noninduced rat was *cis*-nicotine-N'-oxide. In the Aroclor-induced rat, cotinine metabolites were the major metabolites found. Sex differences were found for the urinary nicotine metabolite profile, mainly expressed in the excretion of *cis*-nicotine-N'-oxide, 29% in the male and 17% in the female noninduced rat, and the excretion of cotinine, 5% in the male and 12% in the female noninduced rat. High stereoselectivity was found for the formation of the *cis/trans*-diastereomers of nicotine-N'-oxide as well as of 3'-hydroxycotinine, the stereoselectivity being more pronounced in male rats.

INTRODUCTION

Nicotine metabolism has been extensively studied, and more than 20 nicotine metabolites resulting from 5 different metabolic pathways have been described [for review, *see* (1–5)]. The metabolism of the naturally occurring S-enantiomer and of the synthetic R-enantiomer of nicotine were shown to be different in vitro (6) as well as in vivo (7), and one enantiomer was found to interfere with the metabolism of the other (8). In the aforementioned in vivo metabolism study in the rat using enantiomerically pure nicotine tritiated at the methyl group (7), only a limited number of metabolites could be detected. This is because the methyl group is lost at early steps in the metabolic pathways. Therefore, the present study was performed using the enantiomerically pure S-nicotine, [^{14}C]-labeled at the 2'-position in the pyrrolidine ring.

It is known that the metabolism and biokinetics of nicotine are influenced by genetic (e.g. strain, sex) and exogenous factors (e.g. drug treatment, diet) [reviewed in (5)], which can affect the induction status of the metabolizing enzymes. The influence of selective inducers of various cytochrome P-450 isozymes, e.g. phenobarbital, 5,6-benzoflavone, β-naphthoflavone, 3-methylcholanthrene, or ethanol on nicotine metabolism has been shown in vitro on subcellular fractions or isolated organs [e.g. (9–15)]. In vivo, the influence of

Please send reprint requests to: Dr Georg Schepers, INBIFO Institut für biologische Forschung, Fuggerstr. 3, D-51149 Köln, Germany

selective cytochrome P-450 isozyme induction on nicotine metabolism in the rat was shown using racemic nicotine (16, 17).

To our knowledge, no in vivo study with nicotine has been performed using a nonselective inducer for various enzyme systems, e.g. Aroclor 1254, a mixture of polychlorinated biphenyls commonly used for the preparation of the postmitochondrial (S9) fraction for metabolizing promutagens in in vitro assays (18). The influence of such a nonselective inducer on nicotine metabolism, however, is of special interest since nicotine metabolism includes not only P-450-related but also other enzymatic pathways.

In the present study, the in vivo metabolism and biokinetics of S-[pyrrolidine-2'-^{14}C]-nicotine as well as the influence of enzyme induction by Aroclor 1254 are presented in detail for male and female rat. In addition, a comprehensive analysis of major and minor urinary nicotine metabolites, based on the completely recovered radioactivity, is given.

MATERIALS AND METHODS

Test substance

S-[Pyrrolidine-2'-^{14}C]-nicotine-di-L-tartrate salt (^{14}C-NIC) was synthesized by Chemsyn Science Laboratories (Lenexa, Kans, USA): specific radioactivity: 58.8 mCi/mmol, chemical purity: > 97%, radiochemical purity: > 98%, optical purity: > 98%. The optical purity was confirmed by determining the enantiomeric excess by HPLC (19) and GC on chiral columns (publication in preparation). The ^{14}C-NIC was dissolved in phosphate-buffered saline (phosphate 10 mmol/l, pH 7.2, NaCl 140 mmol/l) to a final activity of 2.5 mCi/ml, and aliquots of this stock solution were stored at –80°C.

Reference compounds

S-nicotine (NIC) was from Sigma (Deisenhofen, Germany); cotinine (COT) from Roth (Karlsruhe, Germany); 3-pyridylacetic acid (PAAc) from Aldrich (Steinheim, Germany). Dihydrometanicotine (DHMNIC), *cis*-3'-hydroxycotinine (CHOC), *trans*-3'-hydroxycotinine (THOC), cotinine-N-oxide (CNO), norcotinine (NCOT), nornicotine (NNIC), 4-(3-pyridyl)-4-oxo-N-methyl-butyramide (POMBAm), 4-(3-pyridyl)-4-oxobutyric acid (POBAc), N,N'-dimethylnicotinium diiodide (NN'DMN), 4-(3-pyridyl)-4-methyl-aminobutyric acid (PMABAc), 4-(3-pyridyl)-4-aminobutyric acid (PABAc), 4-(3-pyridyl)-butyric acid (PBAc), 4-(3-pyridyl)-4-hydroxybutyric acid (PHOBAc), and 5-(3-pyridyl)-tetrahydrofuranone (PTHF) were from Dr G. Neurath (Institut für Biopharmazeutische Mikroanalytik, Hamburg, Germany). 5'-Hydroxycotinine (5HOC), demethylcotinine-N-oxide (DMCNO), and the methyl ester of POBAc were from Philip Morris (Richmond, Va, USA); 1'(R)-2'(S)-*cis*- and 1'(S)-2'(S)-*trans*-nicotine-N'-oxide (CNN'O and TNN'O) were from Prof. J. Gorrod (Kings College, London, UK); N-methylnicotinium iodide (NMNIC), N'-methylnicotinium iodide (N'MNIC), N-methylcotininium iodide (NMCOT), and N-methyl-N'-oxonicotinium iodide (NMNN'O) were from Prof. P. Crooks (University of Kentucky, Lexington, USA). For the structural formulae of nicotine metabolites and reference compounds, *see* Figure 1.

Other chemicals

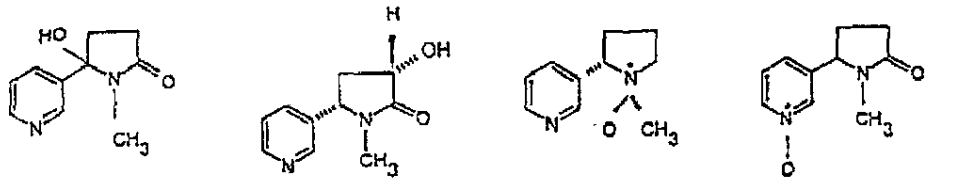
Aroclor 1254 was from Analabs (via Antechnica, Etlingen, Germany) and was dissolved in corn oil (200 g/l). All other chemicals were from commercial sources and of the highest purity available.

Animals

Male and female Sprague Dawley CrL:CD(SD)BR outbred albino rats (Charles River Wiga, Sulzfeld, Germany) were used. 14 days before administration of ^{14}C-NIC, a vascular access port (Implantofix-R, Braun Melsungen, Melsungen, Germany) was subcutaneously implanted with access to the vena jugularis (20). The rats were individually housed in polycarbonate cages with bedding material. During the whole study, water and diet (HERILAN MRH-HALTUNG, Eggersmann, Rinteln, Germany) were provided *ad libitum*. The room temperature was maintained at 22 ± 2°C. The light/dark cycle was 12:12 h, the light cycle starting at 06.00 am.

Administration of Aroclor

Those rats used to study the influence of enzyme induction on the metabolism and biokinetics of NIC received a single i.p. dose of Aroclor 1254 (0.5 g/kg) 5 days prior to ^{14}C-NIC administration. The protocol was the same as that used for the preparation of the postmitochondrial fraction for metabolizing promutagens in in vitro assays (18).

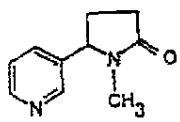


5HOC

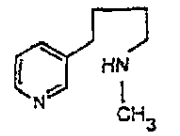
CHOC

CNN'O

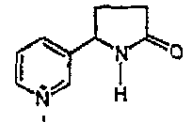
CNO



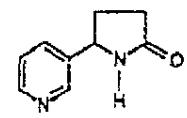
COT



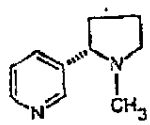
DHMNIC



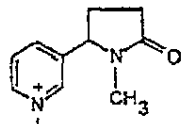
DMCNO



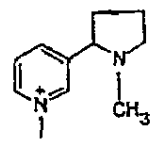
NCOT



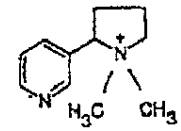
NIC



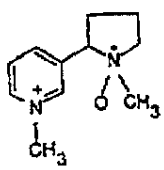
NMCOT



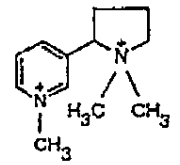
NMNIC



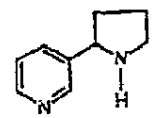
N'MNIC



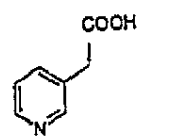
NMNN'O



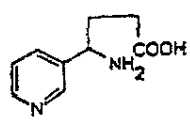
NN'DMNIC



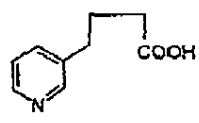
NNIC



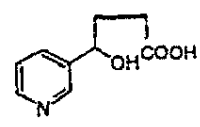
PAAc



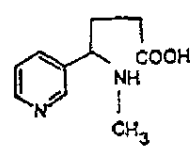
PABAc



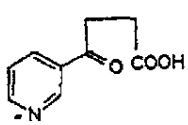
PBAc



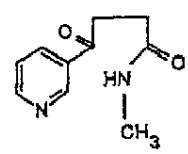
PHOBAc



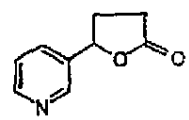
PMABAc



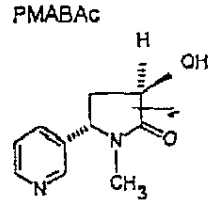
POBAc



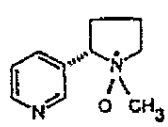
POMBAm



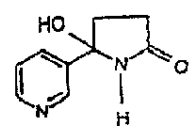
PTHF



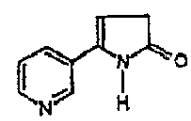
THOC



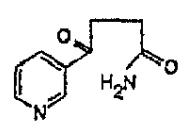
TNN'O



Allohydroxy-demethylcotinine



Demethylcotinine-$\Delta^{4',5'}$-enamine



4-(3-Pyridyl)-4-oxo-butyramide

Fig. 1: Structural formulae of nicotine metabolites and reference compounds.

Administration of S-nicotine

The ^{14}C-NIC stock solution was diluted with physiological saline to a final concentration of 0.9 µmol/ml. The rats (230–300 g body weight) received an i.v. dose of 4.6 µmol ^{14}C-NIC/kg (0.75 mg nicotine base/kg) at a rate of 13.3 µl/(kg· s) by a motor-driven Hamilton syringe via the implanted vascular access port. Following ^{14}C-NIC administration, the rats were housed in metabolism cages for up to 96 h.

Collection of urine and feces

Urine was collected in 50 ml ice-cooled polypropylene tubes preloaded with 200 µl of L-tartaric acid (5 mol/l). Feces were collected in ice-cooled glass containers. The surfaces of the metabolism cages were rinsed with water and the wash water collected. The urine samples were centrifuged and stored at –20 °C until chromatographic analysis.

Determination of radioactivity

Radioactivity was determined by liquid scintillation counting (LSC) using a TRI-CARB 2550 TR liquid scintillation analyzer (Canberra-Packard, Frankfurt, Germany). Urine and wash water samples were prepared directly in Aquasafe 300 (Zinsser Analytic, Frankfurt, Germany). Feces and tissue samples were homogenized and the radioactivity was determined following oxidative combustion in an OX500 sample oxidizer (Harvey Instruments, via Zinsser Analytic, Frankfurt, Germany), by trapping the CO_2 in Oxysolve C-400 (Zinsser Analytic, Frankfurt, Germany).

Radiochromatography

HPLC of the urine samples was performed using a Hewlett Packard 1090 L liquid chromatograph (Waldbronn, Germany) equipped with a ternary solvent delivery system and a diode array detector (10 µl cell) connected in series to a RAMONA 90 radioactivity detector (150 µl calcium fluoride solid scintillator flow cell, Raytest, Straubenhardt, Germany). The analog signals of the UV detector (254 nm) and of the radioactivity detector were evaluated by calculating peak areas using a chromatography data system (Multichrom, VG Instruments, Wiesbaden, Germany). Peak identification was performed by comparison with the retention times of the reference compounds. Radio-HPLC was performed by ion-pair chromatography on two analytical Nova-Pak C18 columns connected in series. A nonlinear ternary solvent program was applied. The mobile phase consisting of a potassium phosphate buffer (pH 3.6), containing pentane-, heptane-, and octane-sulfonic acid, and acetonitrile was pumped at a flow rate of 0.6 ml/min. A typical radiochromatogram is shown in Figure 2. To verify the results obtained by ion-pair chromatography, a cation-exchange HPLC method based on the method of Cundy and Crooks (21) was used following modifications. Full details of both HPLC methods are described by Demetriou et al. (22).

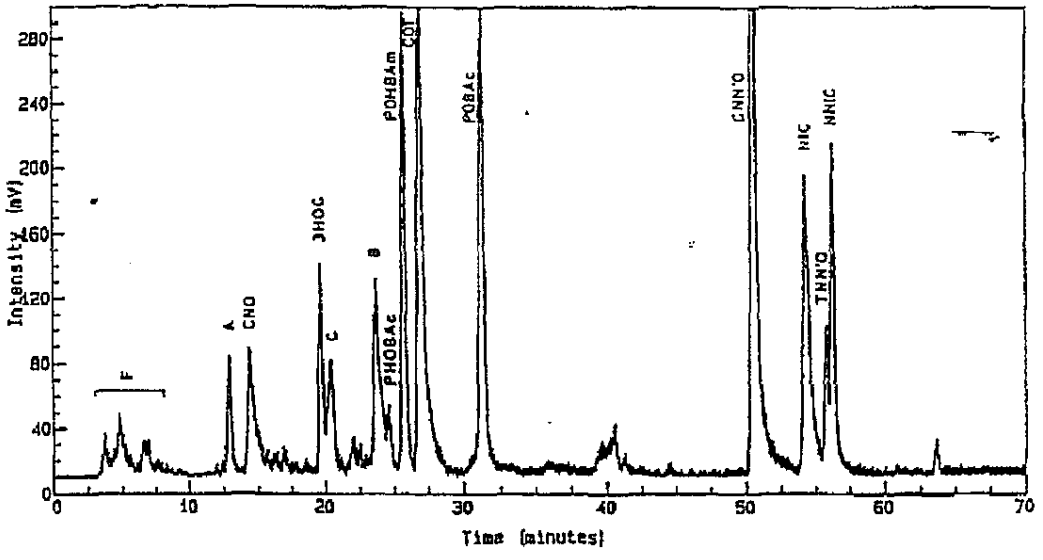


Fig. 2: Ion-pair radio-HPLC of a 24 h urine sample from a noninduced female rat dosed with 2′-[^{14}C]-labeled S-nicotine.

Evaluation of the urinary excretion kinetics

To evaluate the urinary excretion kinetics of NIC and its major metabolites, sigma minus plot analysis was used (23): the difference between the total amount of a metabolite excreted (U_∞) and the amount excreted up to a given time point (U_t) relative to the administered NIC was plotted logarithmically versus time.

RESULTS AND DISCUSSION

Total excretion and recovery of the administered radioactivity

Rats dosed with ^{14}C-NIC excreted 95% of the administered radioactivity in urine within 96 h. The excretion in feces was 4% and the radioactivity determined in the body 1%. The exhaled radioactivity was determined to be less than 0.1%. Hence, complete recovery of the administered radioactivity was achieved. The results were the same for both noninduced and Aroclor-induced rats.

Profile of urinary metabolites

More than 40 peaks were found in the radiochromatograms of the urine samples; 18 of them coincided with chromatographic properties of authentic synthetic compounds. For the diastereomers of nicotine-N'-oxide, CNN'O and TNN'O, baseline separation was achieved with both ion-pair and ion-exchange HPLC. However, due to insufficient separations THOC and CHOC were determined in sum and designated as 3'-hydroxycotinine (3HOC). Furthermore, PMABAc and its lactam COT as well as 5HOC and its ring-opened tautomer POMBAm were not separated. They were designated as COT and POMBAm, respectively.

When considered according to the amounts excreted in the urine of noninduced male rats within 24 h following the administration of NIC, the first 20 metabolites accounted for more than 90% of the excreted radioactivity (Fig. 3). Of these 20 metabolites, 12 were identified. Unidentified NIC metabolites represented 20% of the radioactivity excreted in the urine of noninduced and 35% of the radioactivity excreted in the urine of the Aroclor-induced rats.

The major urinary NIC metabolite in the noninduced male rat is CNN'O (29%), followed by NIC (11 %) and by POBAc (10%) (Fig. 3a). COT accounted for 5% of the administered radioactivity. The NIC found may have been eliminated either unmetabolized or after metabolic formation followed by the reduction of nicotine-N'-oxide (6, 24–27). In the metabolite profile of the Aroclor-induced male rats, the formation of secondary metabolites via COT predominated, thus indicating a shift from the N- to the C-oxidation pathway. The major metabolites in the induced rat were CNO (14%), POBAc (12%), POMBAm (9%), and 3HOC (7%), whereas CNN'O decreased to 5% (Fig. 3b). COT accounted for less than 2% of the

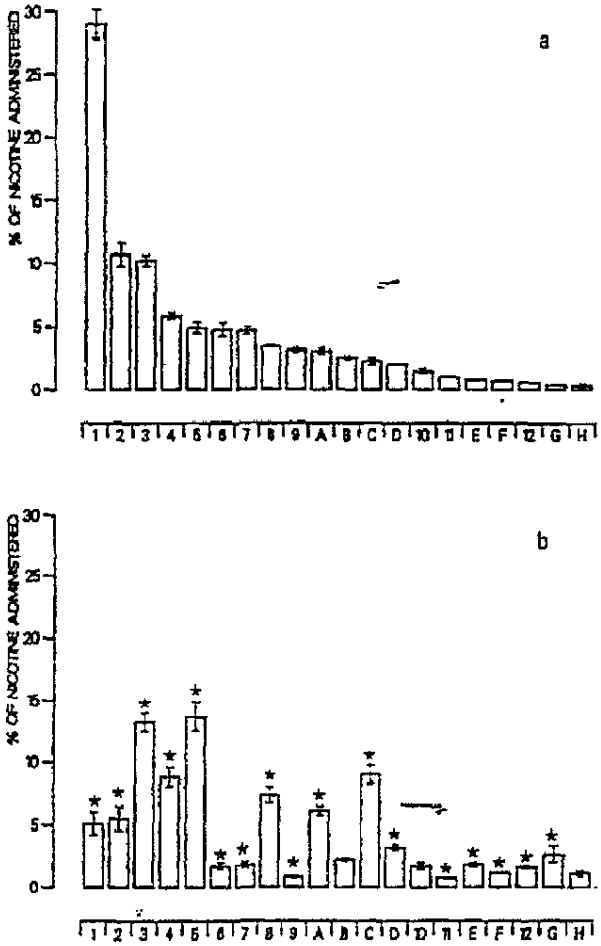


Fig. 3: Profile of urinary nicotine metabolites excreted over 24 h after i.v. S-nicotine administration to male rats. a: noninduced (M ± SE, n = 5); b: Aroclor-induced (M ± SE, n = 5). 1: CNN'O; 2: NIC; 3: POBAc; 4: POMBAm; 5: CNO; 6: COT; 7: NNIC; 8: 3HOC; 9: TNN'O; 10: PHOBAc; 11: NCOT; 12: PAAc; A to H: unknown (F: phase 2 metabolites?). Statistically significant differences ($P < 0.05$) between noninduced and Aroclor-induced rats are indicated by stars.

administered radioactivity. For NIC and most of its metabolites, statistically significant differences were seen in the amounts excreted in the urine between noninduced and Aroclor-induced rats.

Of the metabolites not shown in Figure 3, each contributed to less than 1% of the administered radioactivity. These included the following identified metabolites NMNN'O, NN'DMNIC, PTHF, PABAc, N'MNIC, and PBAc, accounting in total for about 1% of the administered radioactivity.

A comparison of the chromatograms of the urine samples with those of the reference compounds did not indicate that DHMNIC, NMNIC, NMCOT, and DMCNO are urinary S-nicotine metabolites in the rat.

The urinary metabolite profile of R-nicotine in the rat is different from that of S-nicotine as indicated by results we have obtained in an ongoing study. Differences were pronounced, e.g. in the excretion of TNN'O, NNIC, and COT as the major R-nicotine metabolites in the noninduced rat. These results as well as the fact that S-nicotine interferes with the metabolism of R-nicotine (8) make it questionable to compare the metabolite profile obtained for S-nicotine in our study with that previously published for racemic R/S-nicotine (28).

Unidentified metabolites

Of the unidentified metabolites, those designated as B, C, and D showed different peak shapes in the chromatograms of different urine samples. This led us to the assumption that each of these peaks represents more than one metabolite. The formation of metabolite C, eluting immediately after 3HOC, was significantly increased by Aroclor induction. Metabolite A, which was also significantly enhanced by induction, is probably strongly polar as indicated by its early elution in front of CNO under both ion-pair and ion-exchange chromatographic conditions. Using ion-pair HPLC, metabolite A was found to have the same retention time as DMCNO, which has been proposed to be a nicotine metabolite (29). However, due to the different retention times of metabolite A and the reference compound DMCNO in the ion-exchange HPLC, it can be concluded that DMCNO is not a NIC metabolite in the rat.

Metabolites were observed that elute at the beginning of the chromatograms (designated together as F). This indicates that they are even more polar than metabolite A. Therefore, we assumed that they represent the nicotine phase 2 metabolites which have been found in human urine (30–35) as well as in the urine of NIC-treated rats and hamsters (34).

Recently, allohydroxydemethylcotinine has been described as a nicotine metabolite found in the urine of rats (36), and its dehydration product demethylcotinine-$\Delta^{4',5'}$-enamine in the urine of humans (32). In order to check whether these metabolites occur in the urine of NIC-treated rats, we performed a synthesis of allohydroxydemethylcotinine according to Kyeremateu by ammonolysis of the methyl ester of POBAc. Ion-pair HPLC of the reaction mixture yielded 2 major product peaks. GC/MS analysis of each of these 2 HPLC fractions yielded similar results: for each fraction 2 peaks were found by GC, one showing the mass spectrum identical with that of 4-(3-pyridyl)-4-oxobutyramide (37), the other a mass spectrum most probably of demethylcotinine-$\Delta^{4',5'}$-enamine. 4-(3-Pyridyl)-4-oxobutyramide is the ring-opened tautomer of allohydroxydemethylcotinine and therefore – analogous to 5HOC and POMBAm – we do not expect that our HPLC method is able to separate these 2 compounds. Demethylcotinine-$\Delta^{4',5'}$-enamine is the dehydration product of these 2 compounds. However, neither of the 2 product peaks in the HPLC corresponded to any of the peaks found in the radiochromatograms of the rat urine samples. Therefore, we conclude that neither allohydroxydemethylcotinine, 4-(3-pyridyl)-4-oxobutyramide, nor demethylcotinine-$\Delta^{4',5'}$-enamine are urinary metabolites of NIC in the rat.

Kinetics of urinary excretion

The sigma minus plots of NIC and its major urinary metabolites (Fig. 4) are not linear over the full time range but have three different curve shapes. The concave curves of NIC and CNN'O are linear during the first 6 h. For this period, the urinary excretion half-times ($t_{1/2}$) were calculated to be 1.6 h for NIC and 2.1 h for CNN'O in the noninduced and 0.9 and 1.3 h, respectively, in the Aroclor-induced male rats. For the other urinary metabolites in Figure 4, convex curves in the noninduced and sigmoidal curves in the induced rats were observed. This was the case not only for the secondary metabolites via COT (POBAc, POMBAm, CNO, and 3HOC) and the unidentified metabolite A but also for COT and NNIC. Both COT and NNIC are formed in a two-step reaction via iminium ion intermediates (38, 39) in contrast to CNN'O which is formed by a one-step oxidation of NIC. The fact that the excretion kinetics of the unidentified metabolite A are very similar to those of the secondary metabolites via COT and in addition that its formation is enhanced by Aroclor induction in the same way as the second-

ary metabolites via COT led us to the assumption that metabolite A might be also a secondary metabolite via COT.

The values for the shortest excretion half-time, calculated from the steepest slope in the sigma-minus plots, are shown in Table I. The convex sigma minus

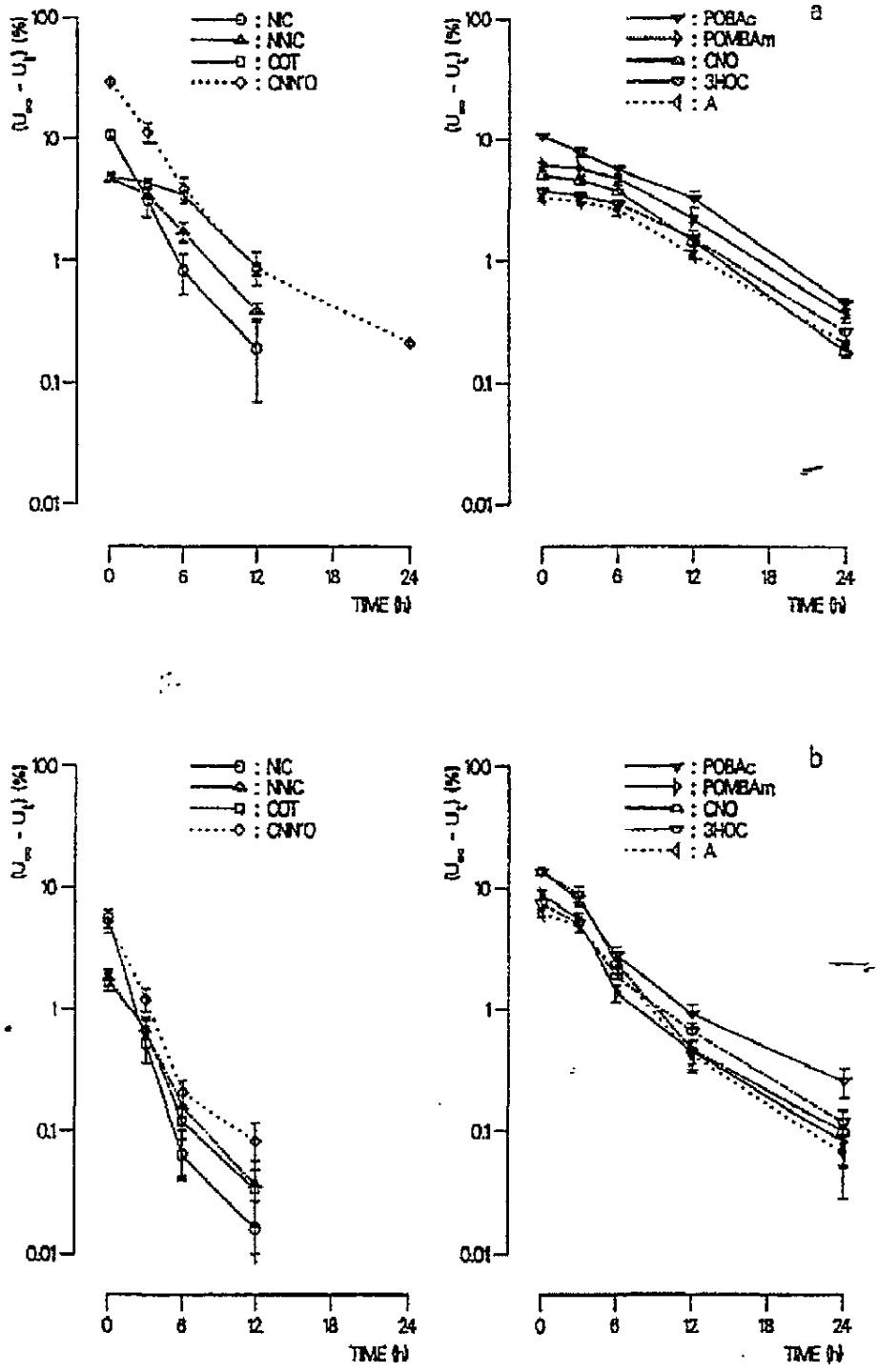


Fig 4: Sigma-minus plots of the urinary excretion kinetics of S-nicotine and metabolites in the male rat. a: noninduced (M ± SE, n = 5); b: Aroclor-induced (M ± SE, n = 5).

Table I: Shortest excretion half-time $t_{1/2}$ of S-nicotine and metabolites in male rats.

Metabolite	$t_{1/2}$ *(h)*	
	Non-induced	*Aroclor induced*
NIC	1.6	0.9
CNN'O	2.1	1.3
NNIC	2.7	1.5
COT	3.1	1.2
POBAc	4.3	1.9
POMBAm	4.8	1.5
CNO	4.0	1.5
3HOC	4.9	2.0
A	5.0	2.6

Calculated from the steepest slope in the sigma-minus plots.

plots can be explained in that the formation rate is more rapid than the elimination rate within the first 3 h. For CNN'O, this period might be missed due to the very fast metabolism of NIC to this primary metabolite and the fast excretion kinetics of CNN'O.

Comparison of the metabolite profiles of male and female rats

In general, NIC metabolism and its inducibility were similar for male and female rats. All urinary metabolites found in the male rat were also found in the female rat. For the female as for the male rat, unidentified metabolites accounted for approx. 20% of the urinary excreted radioactivity in the noninduced and for 35% in the Aroclor-induced rat. Statistically significant differences in the excreted amount of NIC and its metabolites were found between the noninduced and induced female rats (Fig. 5): Aroclor induction leads to a reduction in the formation of CNN'O and to an enhanced formation of secondary metabolites via COT, e.g. POBAc, POMBAm, CNO, and 3HOC, as it was also found for the male rat.

For some metabolites, however, different urinary excretion was observed between male and female rats. The ratios for the excretion over 24 h and the results of the statistical evaluation of these differences are given in Table II. Major differences were seen between the noninduced male and female rats. The urinary excretion of CNN'O, which for both sexes is the major NIC metabolite, was lower and the excretion of COT was higher in the female rat. The lower N'-oxidation of nicotine observed in the female rats is in accordance with the recently reported sex differences in flavin-containing monooxygenase activity (40). Furthermore, noninduced and Aroclor-induced female rats excrete significantly higher amounts of the unknown metabolites F, which are assumed to represent phase 2 metabolites of NIC, than the male rat.

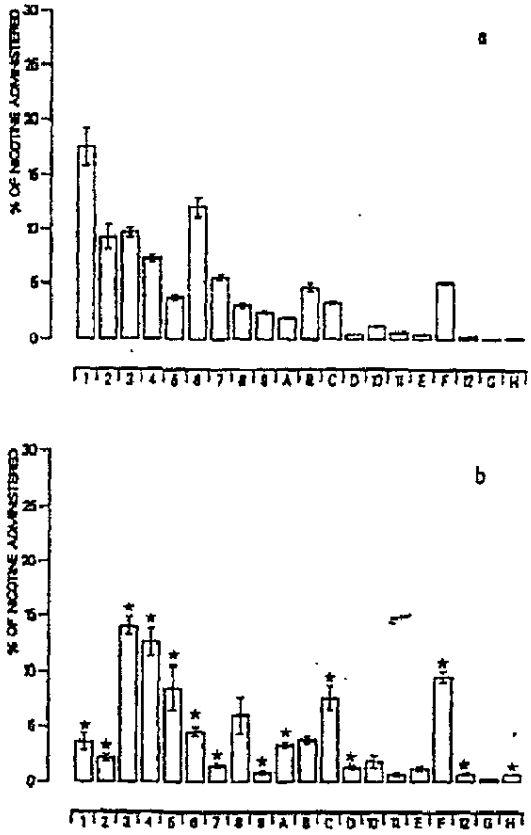


Fig. 5: Profile of urinary nicotine metabolites excreted over 24 h after i.v. S-nicotine administration to female rats. a: noninduced (M ± SE, n = 5): b: Aroclor-induced (M ± SE, n = 4). 1: CNN'O; 2: NIC; 3: POBAc; 4: POMBAm; 5: CNO; 6: COT; 7: NNIC; 8: 3HOC; 9: TNN'O; 10: PHOBAc; 11: NCOT; 12: PAAc; A to H: unknown (F: phase 2 metabolites?). Statistically significant differences ($P < 0.05$) between noninduced and Aroclor-induced rats are indicated by stars.

Stereoselectivity of S-nicotine metabolism

During the formation of nicotine-N'-oxide (NN'O) and 3HOC, two of the major NIC metabolites in the rat, a second stereogenic center in their molecular structure is formed. Therefore, it can be expected that the me-

Table II : Ratio of urinary excretion of S-nicotine and metabolites over 24 h between male and female rats.

Metabolite	*Ratio*	
	Non-induced	*Aroclor induced*
CNN'O	1.7*(a)	1.4
NIC	1.2	2.6*
POBAc	1.0	0.9
POMBAm	0.8*	0.7
CNO	1.3*	1.6
COT	0.4*	0.4*
NNIC	0.8	1.2
3HOC	1.1	1.2
TNN'O	1.3	1.0
A	1.5*	1.8*
B	0.5*	0.6*
C	0.7	1.2
D	4.3*	2.3*
PHOBAc	1.2	0.9
NCOT	1.5*	1.1
E	1.8*	1.5
F	0.1*	0.1*
PAAc	1.9	2.3*
G	2.8*	10.0*
H	1.4	1.7

(a) Statistically significant differences ($P < 0.05$) in the urinary excretion of nicotine metabolites between noninduced male and female rats as well as between Aroclor-induced male and female rats are indicated by asterisks.

tabolism of NIC may lead to the predominant formation of one of the diastereomers of these metabolites.

We found that the in vivo N'-oxidation of NIC in both male and female rats leads predominantly to the formation of the *cis*-diastereomer of NN'O, CNN'O (*see* Table III). This stereoselectivity was statistically significantly more pronounced in the noninduced compared to the Aroclor-induced rats. This highly stereoselective N-oxidation pathway is catalyzed by flavin-containing monooxygenases (41) and has been previously reported for several in vitro studies (6, 42–44).

As already mentioned, separation of the diastereomers of 3HOC was not achieved by the radiochromatographic methods. However, we achieved their separation by HPLC after diethylthiobarbituric acid derivatization (34), and we were able to show that in both male and female rats the *trans*-diastereomer of 3HOC, THOC, is predominantly formed (*see* Table III). This confirms recently reported results (45). The stereoselectivity of the 3'-hydroxylation of COT was statistically significantly higher in the Aroclor-induced compared to the non-induced rats. The stereoselective formation of diastereomers of NN'O as well as of 3HOC was more pronounced in male than in female rats. The differences in stereoselectivity found for the formation of NN'O as well as of 3HOC in the noninduced and Aroclor-induced rats lead to the assumption that different isozymes of the respective enzymes are involved and that these isozymes are induced and/or repressed to a different extent.

CONCLUSION

The results of this study demonstrate that there are considerably more S-nicotine metabolites present in the urine of rats than previously described. Important differences exist in the metabolism and excretion kinetics between noninduced and Aroclor-induced rats and in the urinary excretion of some S-nicotine metabolites between male and female rats. The formation of the *cis/trans*-diastereomers of nicotine-N'-oxide and 3'-hydroxycotinine is highly stereoselective. The results underline the importance of exogenous factors and genetic composition on the complex metabolism and biokinetics of S-nicotine.

ACKNOWLEDGEMENTS

We thank Prof. J. Gorrod and Prof. P. Crooks for the gift of reference compounds, Dr P. Voncken for helpful discussions and for providing the GC-MS analysis, Mr D. Hanselmann for critical reading of the manuscript, and Mrs G. Fleger, Mrs G. Kunz, Mr J. Doll as well as Mr D. Demetriou for their excellent technical assistance.

Table III : Ratio of the urinary 24 h excretion of the *cis/trans*-diastereomers of nicotine-N'-oxide and 3'-hydroxycotinine in the rat (M ± SE).

Metabolite ratio	*Male*		*Female*	
	Noninduced (*n* = 5)	*Aroclor-induced* (*n* = 5)	*Noninduced* (*n* = 4)	*Aroclor-induced* (*n* = 5)
CNN'O/TNN'O	9.4 ± 0.5	6.0 ± 0.5	7.2 ± 0.4	4.4 ± 0.5
THOC/CHOC	7.7 ± 0.4	13.6 ± 1.3	4.6 ± 0.3	9.0 ± 1.3

REFERENCES

1. Gorrod J.W., Jenner P. (1975) : The metabolism of tobacco alkaloids. In: Hayes W.J., ed. Essays in Toxicology, Vol. 6. New York: Academic Press, pp. 35-78.
2. Schievelbein H. (1982) : Nicotine, resorption and fate. Pharmacol. Ther., 18, 233-248.
3. Nakayama H. (1988) : Nicotine metabolism in mammals. Drug Metab. Drug Interact., 6, 95-122.
4. Benowitz N.L., Porchet H., Jacob P. (1990) : Pharmacokinetics, metabolism, and pharmacodynamics of nicotine. In: Wonnacott S., Russell M.A.H., Stolerman I.P., eds. Nicotine Psychopharmacology; Molecular, Cellular, and Behavioural Aspects. Oxford: University Press, pp. 112-157.
5. Kyerematen G.A., Vesell E.S. (1991): Metabolism of nicotine. Drug Metab. Rev., 23, 3-41.
6. Jenner P., Gorrod J.W., Beckett A.H. (1973) : Species variation in the metabolism of *R*-(+)- and *S*-(−)-nicotine by α-C- and N-oxidation in vitro. Xenobiotica, 3, 573-580.
7. Nwosu C.G., Crooks P.A. (1988) : Species variation and stereoselectivity in the metabolism of nicotine enantiomers. Xenobiotica, 18, 1361-1372.
8. Cundy K.C., Crooks P.A., Godin C.S. (1985) : Remarkable substrate-inhibitor properties of nicotine enantiomers towards a guinea pig lung aromatic azaheterocycle N-methyltransferase. Biochem. Biophys. Res. Commun., 128, 312-316.
9. Turner D.M. (1977) : The effects of some tobacco smoke constituents on foreign compound metabolism in the cat and the rat. Res. Commun. Chem. Pathol. Pharmacol., 16, 85-100.
10. McCoy G.D., DeMarco G.J. (1986) : Characterization of hamster liver nicotine metabolism. II. Differential effects of ethanol or phenobarbital pretreatment on microsomal N and C oxidation. Biochem. Pharmacol., 35, 4590-4592.
11. Rödell U., Foth H., Kahl G.F. (1987) : Eightfold induction of nicotine elimination in perfused rat liver by pretreatment with phenobarbital. Biochem. Biophys. Res. Commun., 148, 192-198.
12. Shinegaga M.K., Trevor A.J., Castagnoli N. (1988) : Metabolism-dependent covalent binding of (*S*)-[5-^{3}H]nicotine to liver and lung microsomal macromolecules. Drug Metab. Dispos., 16, 397-402.
13. McCoy G.D., DeMarco G.J., Koop D.R. (1989) : Microsomal nicotine metabolism: A comparison of relative activities of six purified rabbit cytochrome P-450 isozymes. Biochem. Pharmacol., 38, 1185-1188.
14. Kyerematen G.A., Morgan M., Warner G., Martin L.F., Vesell E.S. (1990) : Metabolism of nicotine by hepatocytes. Biochem. Pharmacol., 40, 1747-1756.
15. Foth H., Looschen H., Neurath H., Kahl G.F. (1991) : Nicotine metabolism in isolated perfused lung and liver of phenobarbital- and benzoflavone-treated rats. Arch. Toxicol., 65, 68-72.
16. Adir J., Wildfeuer W., Miller R.P. (1980) : Effect of ethanol pretreatment on the pharmacokinetics of nicotine in rats. J. Pharmacol. Exp. Ther., 212, 274-279.
17. Foth H., Walther U.I., Kahl G.F. (1990) : Increased hepatic nicotine elimination after phenobarbital induction in the conscious rat. Toxicol. Appl. Pharmacol., 105, 382-392.
18. Ames B.N., McCann J., Yamasaki E. (1975) : Methods for detecting carcinogens and mutagens with the salmonella/mammalian-microsome mutagenicity test. Mutat. Res., 31, 347-363.
19. Demetriou D., Rustemeier K., Voncken P., Schepers G. (1993) : HPLC separation of the enantiomers of nicotine and nicotine-like compounds. Chirality, In press.
20. Lehnhardt F.-J. (1989) : A new system for catheterism of the V. cava in rats for longtime infusions (Implantofix-R). Z. Versuchstierkd., 32, 171-178.
21. Cundy K.C., Crooks P.A. (1984) : High-performance liquid chromatographic method for the determination of N-methylated metabolites of nicotine. J. Chromatogr., 306, 291-301.
22. Demetriou D., Rustemeier K., Voncken P., Schepers G. (1992) : Two radiochromatographic HPLC methods for the determination of nicotine metabolites. Med. Sci. Res., 20, 873-875.
23. Kyerematen G.A., Damiano M.D., Dvorchik B.H., Vesell E.S. (1982) : Smoking-induced changes in nicotine disposition: application of a new HPLC assay for nicotine and its metabolites. Clin. Pharmacol., 32, 769-780.
24. Jenner P., Gorrod J.W., Beckett A.H. (1973) : The absorption of nicotine-1'-N-oxide and its reduction in the gastro-intestinal tract in man. Xenobiotica, 3, 341-349.
25. Dajani R.M., Gorrod J.W., Beckett A.H. (1975) : Reduction in vivo of (−)-nicotine-1'-N-oxide by germ-free and conventional rats. Biochem. Pharmacol., 24, 648-650.
26. Dunn M.J., Yu L., Savvaspridi C., Jacob III P., Benowitz N.L. (1991) : Disposition kinetics and metabolism of nicotine-1'-N-oxide in rabbits. Drug Metab. Dispos., 19, 667-672.
27. Sepkovic D.W., Haley N.J. (1987) : Metabolism of nicotine in smokers and non-smokers. In: Martin W.R., Van Loon G.R., Iwamoto E.T., Davis L., eds. Tobacco Smoking and Nicotine. New York: Plenum Press, pp. 375-388.
28. Kyerematen G.A., Owens G.F., Chattopadhyay B., deBethizy J.D., Vesell E.S. (1988) : Sexual dimorphism of nicotine metabolism and distribution in the rat. Drug Metab. Dispos., 16, 823-828.
29. Castro A., Monji N., Ali H., Yi J.M., Bowman E.R., McKennis H. (1980) : Nicotine antibodies: Comparison of ligand specificities of antibodies produced against two nicotine conjugates. Eur. J. Biochem., 104, 331-340.
30. Curvall M., Kazemi Vala E., Englund G., Enzell C.R. (1989): Urinary excretion of nicotine and its major metabolites. Paper No. 54 presented at the 43rd Tobacco Chemists' Research Conference, Richmond VA., USA.
31. Curvall M., Kazemi Vala E., Englund G. (1991) : Conjugation pathways in nicotine metabolism. In: Adlkofer F., Thurau K., eds. Effects of Nicotine on Biological Systems. Basel: Birkhäuser Verlag, pp. 69-75.
32. Kyerematen G.A., Morgan M.L., Chattopadhyay B., deBethizy J.D., Vesell E.S. (1990) : Disposition of nicotine and eight metabolites in smokers and nonsmokers: Identification in smokers of two metabolites that are longer lived than cotinine. Clin. Pharmacol. Ther., 48, 641-651.
33. Byrd G.D., Chang K.-M., Greene J.M., deBethizy J.D. (1992) : Evidence for urinary excretion of glucuronide conjugates of nicotine, cotinine, and *trans*-3'-hydroxycotinine in smokers. Drug Metab. Dispos., 20, 192-197.
34. Rustemeier K., Demetriou D., Schepers G., Voncken P. (1993) : High-performance liquid chromatographic determination of nicotine and its urinary metabolites via their 1,3-diethyl-2-thiobarbituric acid derivatives. J. Chromatogr., 613, 95-103.
35. Schepers G., Demetriou D., Rustemeier K., Voncken P., Diehl B. (1992) : Nicotine phase 2 metabolites in human urine – structure of metabolically formed *trans*-3'-hydroxycotinine glucuronide. Med. Sci. Res., 20, 863-865.
36. Kyerematen G.A., Taylor L.H., deBethizy J.D., Vesell E.S. (1988) : Pharmacokinetics of nicotine and 12 metabolites in the rat. Drug Metab. Dispos., 16, 125-129.
37. Pilotti A., Enzell C.R., McKennis H., Bowman E.R., Dufva E., Holmstedt B. (1976) : Studies on the identification of tobacco alkaloids, their mammalian metabolites and related compounds by gas chromatography – mass spectrometry. Beitr.

Tabakforsch., 8, 339-349.
38. Murphy P. (1973) : Enzymatic oxidation of nicotine to nicotine $\Delta^{1'(5')}$ iminium ion. J. Biol. Chem., 248, 2796-2800.
39. Nguyen T.-L., Gruenke L.D., Castagnoli N. (1979) : Metabolic oxidation of nicotine to chemically reactive intermediates. J. Med. Chem., 22, 259-263.
40. Lemoine A., Williams D.E., Cresteil T., Leroux J.-P. (1991) : Hormonal regulation of microsomal flavin-containing monooxygenase: Tissue-dependent expression and substrate specificity. Mol. Pharmacol., 40, 211-217.
41. Ziegler D.M. (1980) : Microsomal flavin-containing monooxygenase: Oxygenation of nucleophilic nitrogen and sulfur compounds. In: Jacoby W.B., ed. Enzymatic Basis of Detoxication, Vol. 1. New York: Academic Press, pp. 201-227.
42. Damani L.A., Pool W.F., Crooks P.A., Kaderlik R.K., Ziegler D.M. (1988) : Stereoselectivity in the N'-oxidation of nicotine isomers by flavin-containing monooxygenase. Mol. Pharmacol., 33, 702-705.
43. Booth J., Boyland E. (1970) : The metabolism of nicotine into two optically-active stereoisomers of nicotine-1'-oxide by animal tissues in vitro and by cigarette smokers. Biochem. Pharmacol., 19, 733-742.
44. Jenner P., Gorrod J.W., Beckett A.H. (1971) : Comparative C- and N-oxidation of (+)- and (−)-nicotine by various species. Xenobiotica, 1, 497-498.
45. Vonckeu P., Rustemeier K., Schepers G. (1990) : Identification of *cis*-3'-hydroxycotinine as a urinary nicotine metabolite. *Xenobiotica, 20, 1353-1356.*